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<b>(21) International Application Number:</b> PCT/US89/01673 <b>(22) International Filing Date:</b> 20 April 1989 (20.04.89)  <b>(30) Priority data:</b> 183,838 20 April 1988 (20.04.88) US  <b>(71) Applicant:</b> THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US).  <b>(72) Inventors:</b> KRAMER, Fred, R. ; 561 West 231 Street, New York, NY 10463 (US). LIZARDI, Paul, M. ; 99 Calle Cerritos, Esquina Privada Cerritos, Colonia Rancho Cortes, 62120 Cuernavaca (MX).		<b>(74) Agent:</b> WHITE, John, P.; Cooper & Dunham, 30 Rockefeller Plaza, New York, NY 10112 (US).  <b>(81) Designated States:</b> AU, DK, FI, JP, NO.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> REPLICATABLE HYBRIDIZABLE RECOMBINANT RNA PROBES AND METHODS OF USING SAME  <b>(57) Abstract</b>  The present invention provides a replicable and hybridizable recombinant single-stranded RNA probe molecule comprising: a recognition sequence for the binding of an RNA-directed RNA polymerase; a sequence required for the initiation of product strand synthesis by the polymerase; and a heterologous RNA sequence inserted at a specific site in the internal region of the recombinant molecule and complementary to an oligo- or polynucleotide of interest. This invention also provides methods for determining the presence or concentration of an oligo- or polynucleotide of interest in a sample and for simultaneously determining the presence or concentration of several different oligo- or polynucleotides of interest in a sample.		

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REPLICATABLE HYBRIDIZABLE RECOMBINANT RNA PROBES AND  
METHODS OF USING SAME

5 This application is a continuation-in-part of U.S.  
Application Serial No. 183,838, filed April 20, 1988,  
U.S. Application Serial No. 852,692, filed April 16,  
1986, and U.S. Application Serial No. 614,350, filed  
10 May 25, 1984, the contents of which are herein  
incorporated by reference.

This invention described herein was made in the course  
of work under Grant No. DMB-86-16429 from the National  
Science Foundation, U.S. Department of Health and Human  
15 Services. The U.S. Government has certain rights in  
this invention.

Background of the Invention

20 Throughout this application, various publications are  
referenced and citations provided for them. The dis-  
closure of these publications in their entireties are  
hereby incorporated by reference into this application  
to more fully describe the state of the art to which  
25 this invention pertains.

It is now a well established fact that all living  
organisms including infectious agents, e.g., viruses,  
contain DNA, or sometimes RNA, molecules which carry  
30 genetic information in the form of a nucleotide  
sequence code. While certain segments of this code are  
shared by many organisms, there are other segments  
which contain nucleotide sequences that are unique for  
a particular organism. These sequences are said to be  
35 species-specific and provide a convenient tag or  
footprint that can be utilized for identification of

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that organism. The technique of nucleic acid hybridization (1) has great potential for the rapid detection and typing of infectious agents. However, current hybridization assays have not yet attained the sensitivity and speed required for practical diagnostic use. It has recently been proposed that the sensitivity and speed of bioassays could be improved by linking a replicatable RNA to a hybridization probe (2). After hybridization, the replicatable RNA would be amplified by incubation with the RNA-directed RNA polymerase, Q $\beta$  replicase (3). The enormous number of RNA copies that would be synthesized would serve as a signal that hybridization had occurred. The synthesis of novel nucleic acid hybridization probes are reported in this invention that combine in a single RNA molecule the dual functions of probe and amplifiable reporter.

A distinguishing feature of RNA synthesis by Q $\beta$  replicase is that a small number of template strands can initiate the synthesis of a large number of product strands (4). Million-fold increases in the amount of RNA routinely occur in vitro (5) as a result of an autocatalytic reaction mechanism (6,7): single-stranded RNAs serve as templates for the synthesis of complementary single-stranded products; after the completion of product strand elongation, both the product and the template are released from the replication complex (8); and both strands are free to serve as templates in the next round of synthesis. Consequently, as long as there is an excess of replicase, the number of RNA strands increases exponentially. After the number of RNA strands equals the number of active replicase molecules, RNA synthesis continues linearly. There are a number of advantages to using the amplification of

RNA by Q $\beta$  replicase as the basis of a signal-generating system: Q $\beta$  replicase is highly specific for its own template RNAs (9); as little as one molecule of template RNA can, in principle, initiate replication (10); and the amount of RNA synthesized (typically, 200 ng in 50  $\mu$ l in 15 minutes) is so large that it can be measured with the aid of simple colorimetric techniques.

Two developments led to the current application: 1) the discovery that heterologous RNA segments can be inserted within the sequence of a small, naturally occurring template for Q $\beta$  replicase, MDV-1 RNA (11), without affecting its replicability (12); and 2) the construction of a plasmid that serves as a template for the synthesis of MDV-1 (+) RNA when the plasmid is incubated in vitro with bacteriophage T7 RNA polymerase (13).

This plasmid has been modified in the present invention by inserting a polylinker within the MDV-1 cDNA sequence, and then inserting synthetic hybridization probe sequences within the polylinker. The resulting plasmids served as templates for the synthesis of "recombinant RNAs," which consist of a probe sequence embedded within the sequence of MDV-1 (+) RNA. The probe sequences employed in this invention and which will be further described in the Experimental Details section to follow, are known to hybridize specifically to the repetitive DNA of Plasmodium falciparum (14-16), one of the protozoans that cause malaria. In this invention, it will be shown that these recombinant RNA molecules are bifunctional, in that they are able to hybridize specifically to complementary DNA targets and they are also able to serve as templates for exponential amplification by Q $\beta$  replicase.

In Kramer et al., U.S. Patent No. 4,786,600, issued November 22, 1988, there are disclosed replicatable recombinant single-stranded RNA molecules comprising a recognition sequence for the binding of an RNA-directed RNA polymerase, a sequence for the initiation of product strand synthesis by the polymerase and a heterologous sequence of interest derived from a different RNA molecule inserted at a specific site in the internal region of the recombinant molecule. Kramer et al. does not teach or suggest that if the inserted sequence is a hybridization probe sequence, that the resulting molecules can be replicated after hybridization to produce multiple copies for detection.

In Chu et al., U.S. Application Serial No. 852,692, filed April 16, 1986, methods are disclosed for determining the presence of targets, i.e., analytes, by linking a replicatable RNA, which serves as a reporter group, to a probe, e.g., an oligonucleotide, an antibody or lectin. Chu et al. also disclose that an RNA-directed RNA polymerase can then be used to after hybridization has occurred to produce multiple copies of the replicatable RNA for detection. However, Chu et al. do not describe a method in which different recombinant-RNA "probes" sequences can be used simultaneously in the same assay.

Summary of the Invention

5 The present invention concerns a replicatable and hybridizable recombinant single-stranded RNA probe molecule comprising: a recognition sequence for the binding of an RNA-directed RNA polymerase; a sequence required for the initiation of product strand synthesis by the polymerase; and a heterologous RNA sequence  
10 inserted at a specific site in the internal region of the recombinant molecule and complementary to an oligo- or polynucleotide of interest.

15 The invention also provides a method for determining the presence or concentration of an oligo- or polynucleotide of interest in a sample, comprising the steps of: (a) forming a specific complex between the recombinant-RNA probe molecule of claim 1 and the oligo- or polynucleotide of interest, by incubating  
20 the sample with the recombinant-RNA probe molecules under suitable conditions and for a sufficient period of time to permit complementary nucleotide sequences to hybridize; (b) removing unhybridized recombinant-RNA probe molecules from the reaction mixture; (c) incubating the reaction mixture with an RNA-directed RNA  
25 polymerase capable of synthesizing additional copies of the recombinant-RNA probe molecules that are hybridized to the oligo- or polynucleotide of interest; and (d) detecting the recombinant-RNA probe molecules synthesized in step (c), thereby determining the presence or concentration of the oligo- or polynucleotide of  
30 interest.

35 The invention herein further provides a method for simultaneously determining the presence or concentration of several different oligo- or polynucleotides of

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interest in a sample, comprising the steps of: (a) forming specific complexes between a mixture of different types of recombinant-RNA probe molecules of claim 1, each type having a different inserted sequence, and the oligo- or polynucleotides of interest, by incubating the sample with the mixture of recombinant-RNA probe molecules under suitable conditions and for a sufficient period of time to permit complementary nucleotide sequences to hybridize; (b) removing unhybridized recombinant-RNA probe molecules from the reaction mixture; (c) incubating the reaction mixture with an RNA-directed RNA polymerase capable of synthesizing additional copies of the recombinant-RNA probe molecules which are hybridized to the oligo- or polynucleotides of interest; (d) separating the mixture of synthesized recombinant-RNAs by hybridizing them to an ordered array of polynucleotides bound to a membrane, where each of the polynucleotides is complementary to one type of synthesized recombinant-RNA; and (e) detecting the recombinant-RNA probe molecules produced in step (d), thereby determining the presence or concentration of each oligo- or polynucleotide of interest.

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Brief Description of the Figures

5 Figure 1. Structure of plasmid pT7-MDV-poly. The heavy black line represents MDV-1 cDNA. When this plasmid is cleaved with endonuclease Sma I and incubated in vitro with T7 RNA polymerase, the resulting transcripts are replicatable RNAs.

10 Figure 2. Nucleotide sequences of the recombinant transcripts folded into the secondary structures predicted by a computer program to be most stable (41). MDV-fal-un (+) RNA (A) contains a 58-nucleotide insert (shown between the arrows) in place of the 3-nucleotide  
15 segment, AGU, that occurs in natural MDV-1 (+) RNA (42). The secondary structures that the computer program predicts in the region of the recombinant outside the insert are identical to the secondary structures that were experimentally identified in MDV-1 RNA  
20 (36,37), suggesting that the probe sequence would have little effect on the topology of the MDV-1 domain. Bold letters indicate the nucleotides that are complementary to P. falciparum DNA. Figures 2(B) and 2(C) show the probable secondary structure of the inserts present in MDV-fal-st (+) RNA (B) and MDV-poly (+) RNA  
25 (C).

Figure 3. Replication of recombinant RNAs. (A) Polyacrylamide gel electrophoresis showed the relative  
30 mobility of different transcripts: MDV-1 (a), MDV-poly (b), MDV-fal-un (c), and MDV-fal-st (d). The numbers on the side of the panel indicate the length of each transcript (in nucleotides). (B) Q $\beta$  replicase reactions were initiated with 200 fg of each transcript. Kinetic analysis showed that approximately 100,000  
35 copies of each transcript were synthesized in 20 min-

utes. (C) Electrophoretic analysis of the RNA present after 45 minutes showed that the products were replicates of the transcripts. The products were probably one nucleotide longer than the transcripts, since a nonessential, 3'-terminal adenosine is usually added during replication (21).

Figure 4. Effect of initial RNA concentration on the time-course of recombinant RNA synthesis. A series of 25- $\mu$ l Q $\beta$  replicase reactions were initiated with the following quantities of MDV-fal-un RNA: 140 pg, 1.4 pg, 14 fg, 0.14 fg, and 0 fg, which corresponds to  $10^9$ ,  $10^7$ ,  $10^5$ ,  $10^3$ , and 0 molecules of added template. Samples of each reaction were taken every 5 minutes to determine the amount of RNA that had been synthesized. The results demonstrate that the time spent in the exponential phase of synthesis is increased when the initial number of template molecules is decreased (5). Each 100-fold reduction in the number of recombinant molecules used as template resulted in a 3.59 minute delay in achieving saturation (indicating that the RNA population doubled every 32.4 seconds). The electrophoretic mobility of the RNA present in each reaction after 25 minutes (see inset), compared with the mobility of recombinant RNA (278-nucleotides long) and MDV-1 RNA (223-nucleotides long) markers (m), confirmed that the product of reactions initiated with recombinant RNA was recombinant RNA.

Figure 5. Hybridization of recombinant RNAs. 28 ng MDV-poly RNA (a), 75 ng MDV-fal-st RNA (b), and 75 ng MDV-fal-un RNA (c) where hybridized to dot-blot (34) containing either 0.5  $\mu$ g pPFR6 DNA (a plasmid containing 45 copies of the P. falciparum target sequence) or 0.5  $\mu$ g pUC13 DNA (the plasmid vector used to construct

pPFR6). The recombinant RNA hybridized specifically to plasmids containing target sequences. When the amount of both the MDV-fal-un RNA and the DNA was doubled (d),  
5 a marked increase was seen in the amount of RNA that bound to the target DNA.

Figure 6. Linear relationship between the logarithm of the initial number of RNA molecules added to a Q $\beta$  replicase reaction and the amount of product RNA synthesized. The amount of RNA synthesized in 25 minutes in the reactions shown in Figure 4 was plotted against the logarithm of the number of recombinant molecules that had been added initially to each reaction. These  
10 results illustrate how bioassays that utilize replicatable probes would be interpreted: the amount of product RNA would be proportional to the logarithm of the number of probes bound to targets.  
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Figure 7. Autoradiogram of hybridization reactions showing amount of RNA present at each time point.  
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Detailed Description of the Invention

5 This invention concerns a replicatable and hybridizable recombinant single-stranded RNA probe molecule comprising a recognition sequence for the binding of an RNA-directed RNA polymerase; a sequence required for the initiation of product strand synthesis by the polymerase; and a heterologous RNA sequence inserted at  
10 a specific site in the internal region of the recombinant molecule and complementary to an oligo- or polynucleotide of interest.

15 In one embodiment, the recognition sequence for the binding of an RNA-directed RNA polymerase is in an internal region of the recombinant-RNA probe molecule.

20 In another aspect of this invention, the insertion site for the heterologous RNA sequence is not near any sequence required for the binding of the RNA polymerase or for the initiation of product strand synthesis. In a further aspect, such an insert has a minimal affect on the replicability of the molecule. In a still yet further aspect of this invention, the insert in the  
25 recombinant-RNA probe molecule has a minimal effect upon the secondary and tertiary structure of the molecule. In the practice of this invention, the specific insertion site of the recombinant-RNA probe molecule is at a specific nucleotide. In the practice of this  
30 invention the heterologous sequence of interest is inserted between nucleotides 63 and 64.

35 The sequence in the recombinant RNA probe molecules of the present invention which is required for the initiation of product strand synthesis is a cytidine-rich 3'-terminal sequence.

An RNA-directed RNA polymerase useful in the practice of this invention is Q $\beta$  replicase.

5

Useful in to the present invention is a recombinant-RNA probe molecule wherein the molecule is a variant RNA template for Q $\beta$  replicase or a mutant thereof. In a further aspect, such a variant RNA template is MDV-1 RNA or a mutant thereof. In one embodiment, the MDV-1 RNA is MDV-1 (+) RNA. In another, the MDV-1 RNA is MDV-1 (-) RNA.

10

Transcripts derived from a recombinant plasmid by incubation with a DNA-directed RNA polymerase are especially useful in the practice of the invention to provide recombinant-RNA probe molecules. In the practice of this invention, the recombinant-RNA probe molecule is a variant RNA template for Q $\beta$  replicase or a mutant thereof. In a preferred embodiment, the variant RNA template is MDV-1 RNA or a mutant thereof. In still other embodiments, the MDV-1 RNA is MDV-1 (+) RNA or MDV-1 (-) RNA.

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One feature of the invention herein is to provide a recombinant-RNA probe molecule wherein the heterologous sequence is inserted between nucleotides 63 and 64.

25

An important feature is obtained by this invention where the inserted heterologous sequence of the recombinant-RNA probe molecule is complementary to a specific nucleic acid sequence of an infectious agent. Such an infectious agent may be a virus, a viroid or virusoid, a prokaryote, a bacterium, a eukaryote, or a parasitic protozoan, such as the parasitic protozoan that causes malaria.

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5 In a further aspect, the inserted heterologous sequence of the recombinant-RNA probe molecule may be complementary to a specific gene sequence or portion thereof, or to an allele of the specific gene sequence or portion thereof.

10 The invention herein provides a method for determining the presence or concentration of an oligo- or polynucleotide of interest in a sample, comprising the steps of: (a) forming a specific complex between the recombinant-RNA probe molecules, as described above, and the oligo- or polynucleotide of interest, by incubating the sample with the recombinant-RNA probe molecules under suitable conditions and for a sufficient period of time to permit complementary nucleotide sequences to hybridize; (b) removing unhybridized recombinant-RNA probe molecules from the reaction mixture; (c) incubating the reaction mixture with an RNA-directed RNA polymerase capable of synthesizing additional copies of the recombinant-RNA probe molecules that are hybridized to the oligo- or polynucleotide of interest; and (d) detecting the recombinant-RNA probe molecules synthesized in step (c), thereby determining the presence or concentration of the oligo- or polynucleotide of interest.

30 Such a method as described above may be used to generate highly amplified signals in a nucleic acid hybridization assay. In theory the method has the power to generate a signal from a single hybridized molecule, and therefore, could be utilized to devise ultra-high sensitivity DNA (or RNA) detection assay. The method is based on the use of Q $\beta$  RNA recombinant constructs and Q $\beta$  Replicase.

5 In such a method, the oligo- or polynucleotide in the sample may be bound to a solid support. In such cases, the solid support may be a nitrocellulose or nylon membrane.

10 In carrying out the above-described method of this invention and forming a specific complex between the recombinant-RNA probe molecules and the oligo- or polynucleotide of interest, i.e., step (a), the oligo- or polynucleotide of interest and the recombinant-RNA probe molecule may be in solution.

15 The unhybridized recombinant RNA molecules may be separated from those that are hybridized to the oligo- or polynucleotides of interest by employing techniques and skills which are well-known in the art. In the usual case, with the recombinant RNA molecule hybridized to the oligo- or polynucleotide of interest, which in turn is bound to a solid support, such separation is readily accomplished by simple washing which does not significantly disrupt the connection to the solid support. In addition, a technique known as the sandwich hybridization method may also be used to effect separation of hybridized from unhybridized recombinant-RNA molecules. Chromatographic and electrophoretic techniques may be used as well. In a further aspect of the method described hereinabove, the unhybridized recombinant-RNA probe molecules are removed from the reaction mixture in step (b) by separating hybridized recombinant-RNA probe molecules from the unhybridized probe molecules through the capture of the oligo- or polynucleotide onto a solid support.

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In detecting the recombinant-RNA probe molecules which have been synthesized or replicated in step (c) above, methods well-known to those in this art may be employed. For example, detection can be by ultraviolet absorbance of replicated RNA, as, for example, by the method of contact photoprinting (54).

In one embodiment detecting is carried out by the incorporation of radioactively labelled ribonucleoside 5'-triphosphate precursors into the recombinant-RNA products. In another embodiment, detecting is carried out by the incorporation of chemically modified ribonucleoside 5'-triphosphate precursors into the recombinant-RNA products.

Biotin or iminobiotin can be incorporated into replicated RNA, which can then be detected by known techniques with an enzyme-avidin or enzyme-streptavidin adduct, which binds to the RNA-bound biotin and catalyzes production of a conveniently detectable chromogen. See Matthews (55); Leary et al. (45). Incorporation of biotin or iminobiotin into replicated RNA can be accomplished by employing UTP that is biotinylated through a spacer to carbon-5 of the uracil moiety as a substrate for the replicate in the replication reaction. Such UTP's are known compounds. Further, it is known that such UTP's are substrates for Q $\beta$  replicase, and that RNAs which include uracils biotinylated through spacer groups joined to the carbon-5 position, due to use of such UTP's in their synthesis, are templates for Q $\beta$  replicase catalyzed replication.

RNA resulting from the replication process could also be biotinylated employing photobiotin acetate according to the procedure of Forster et al., (56), and then



detected, with an avidin-enzyme adduct-chromogenic compound system, like replicated RNA's synthesized with biotinylated UTP in the replication reaction. Thus, in still another aspect, the chemically modified ribonucleoside 5'-triphosphate precursors may be biotinylated or the chemically modified ribonucleotide 5'-triphosphate precursors may be fluorescent. A further feature of this method is obtained where detecting is carried out by the binding of RNA-specific chromogenic or fluorogenic dyes to the recombinant-RNA products. RNA resulting from the replication process can be made fluorescent by employing a T4 RNA ligase-catalyzed reaction to append nucleotides modified to be fluorescent to the 3'-end of replicative RNA. See Cosstick et al., Nucl. Acis Res. 12, 1791-1810 (57). The fluorescence of the resulting RNA can be employed to detect the RNA by any of the several standard techniques.

Among still other methods that can be used to detect replicated RNA are those wherein a reporter substance, that binds specifically with nucleic acid, is added to the system in which the replication has taken place, or to the medium, such as a positively charged support such as ECTEOLA paper, on which replicated RNA has been isolated, and signal from the reporter substance measured. Such substances include: chromogenic dyes, such as "stains all" (Dahlberg et al., (58); methylene blue (Dingman and Peacock, (59), and silver stain (Sammons et al., 32); Igloie, (60)); fluorogenic compounds that bind to RNA -- for example, ethidium bromide (Sharp et al., (61); Bailey and Davidson, (62); and fluorogenic compounds that bind specifically to RNAs that are templates for replication by Q $\beta$  replicase -- for example, a phycobiliprotein (Oi et al., (63); Stryer et al., U.S. Patent No. 4,520,110) conjugated to the viral

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subunit of Q $\beta$  replicase.

5 Detecting may be also carried out by physical methods, such as the absorption of ultraviolet light and the determination of mass by weighing.

10 In incubating the reaction mixture obtained in step (b), an RNA-directed RNA polymerase is employed. An example of such a polymerase useful in the practice of this invention is Q $\beta$  replicase.

15 This invention also provides recombinant-RNA probe molecules produced by the above-described method and in particular, recombinant-RNA probe molecules produced by a method where the reaction mixture is incubated with Q $\beta$  replicase to synthesize additional copies of the recombinant-RNA probe molecules that are hybridized to the oligo- or polynucleotide of interest.

20 Another aspect of the method provided by this invention is attained when the time of incubation in step (c) is sufficiently short so that the number of recombinant-RNA product strands does not exceed the number of polymerase molecules, with the result that the number of recombinant-RNA product molecules is linearly proportional to the number of recombinant-RNA probe molecules originally hybridized.

30 The method described herein provides another important feature where the time of incubation in step (c) is sufficiently long so that the number of recombinant-RNA product strands exceeds the number of polymerase molecules, with the result that the number of recombinant-RNA product molecules is proportional to the logarithm of the number of recombinant-RNA probe molecules origi-

35

nally hybridized.

5 This invention also provides a method for simultaneous-  
ly determining the presence or concentration of several  
different oligo- or polynucleotides of interest in a  
sample, comprising the steps of: (a) forming specific  
10 complexes between a mixture of different types of re-  
combinant-RNA probe molecules of claim 1, each type  
having a different inserted sequence, and the oligo-  
or polynucleotides of interest, by incubating the sam-  
ple with the mixture of recombinant-RNA probe molecules  
under suitable conditions and for a sufficient period  
15 of time to permit complementary nucleotide sequences to  
hybridize; (b) removing unhybridized recombinant-RNA  
probe molecules from the reaction mixture; (c)  
incubating the reaction mixture with an RNA-directed  
RNA polymerase capable of synthesizing additional  
20 copies of the recombinant-RNA probe molecules that are  
hybridized to the oligo- or polynucleotides of  
interest; (d) separating the mixture of synthesized  
recombinant-RNAs by hybridizing them to an ordered  
array of polynucleotides bound to a membrane, where  
25 each of the polynucleotides is complementary to one  
type of synthesized recombinant-RNA; and (e) detecting  
the recombinant-RNA probe molecules produced in step  
(d), thereby determining the presence or concentration  
of each oligo- or polynucleotide of interest.

30 A good clinical assay requires speed, specificity, and  
sensitivity. For example, current assays for the  
identification of the pathogenic agents that cause  
acute bacterial meningitis lack sufficient speed and  
sensitivity. A number of different organisms are  
35 associated with meningitis, including, Haemophilus  
influenzae, Klebsiella pneumoniae, Neisseria

meningitidis, Staphylococcus aureus, and Streptococcus pneumoniae. The patient is typically a young child. Effective treatment requires the identification of the etiologic agent in a sample of cerebrospinal fluid; and it is essential that antibiotic treatment begin as soon as possible. However, current laboratory techniques require at least 18 hours to identify the agent. Moreover, the sample volume is usually only 50 microliters and often contains less than 50 individual microorganisms, which is well below the limits at which reliable direct detection assays can currently be carried out (64). The clinical picture is further complicated by a marked increase in the abundance of bacteria that possess antibiotic resistance genes on plasmids (65,66).

A series of replicatable recombinant-RNA probes molecules can be prepared each of which is specific for a different organism that can cause meningitis. In addition, a series of recombinant-RNA probe molecules can be prepared, each of which is specific for different antibiotic resistance genes that could be present in the infectious agent.

Using such a series of recombinant-RNA probe molecules a mixture of, for example, 15 different replicatable recombinant-RNA probes are incubated with denatured DNA obtained from a sample of spinal fluid. Only a few types of recombinant-RNA probe molecules species will find targets (for example, one bacterial probe and three resistance gene probes). After removing the unbound probe molecules, Q $\beta$  replicase is used to amplify the remaining probes. After amplification, the mixture of product RNAs is placed in contact with a membrane containing numbered dot-blot, each of which

contains denatured DNA complementary to one of the original probe sequences. In this manner, the mixture of product RNAs is sorted out for subsequent quantification. These assays permit the rapid and simultaneous diagnosis of both the pathogenic agent and its spectrum of antibiotic resistance.

In a similar manner a mixture of probes could be used simultaneously to detect the human immunodeficiency virus which causes acquired immune deficiency syndrome (AIDS) and the concentration of an entire panel of opportunistic agents that infect the immunosuppressed patient.

This invention is illustrated in the Experimental Details and Experimental Discussion sections which follow. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be constructed to, limit in any way the invention as set forth in the claims which follow thereafter.

#### Experimental Details

#### Material and Methods

##### Enzymes

Restriction endonucleases, T4 polynucleotide kinase, and T7 RNA polymerase were purchased from New England Biolabs. Calf intestine alkaline phosphatase, T4 DNA ligase, the Klenow fragment of Escherichia coli DNA polymerase I, and bovine pancreatic deoxyribonuclease I were purchased from Boehringer Mannheim. Q $\beta$  replicase was isolated from bacteriophage Q $\beta$ -infected E. coli Q13 by the procedure of Eoyang and August (17), with the

hydroxylapatite step omitted.

#### Oligonucleotides

5 Single-stranded DNA fragments were prepared, using  $\beta$ -  
cyanoethyl phosphoramidite chemistry (18), in a Micro-  
syn-1450A synthesizer (Systec). After deblocking and  
release from the resin, the oligonucleotides were iso-  
lated by preparative gel electrophoresis (19), eluted  
100 from the gel, filtered through nitrocellulose, and  
purified by chromatography (20) on SEP-PAK C18 car-  
tridges (Waters Associates).

#### Plasmid for Synthesizing MDV-1 (+) RNA by Transcrip- tion

15 pT7-MDV contains a promoter for T7 RNA polymerase di-  
rected towards a full-length cDNA prepared from MDV-1  
RNA (13). This plasmid had been constructed so that  
transcription from the T7 promoter begins with the  
20 first nucleotide of MDV-1 (+) RNA. A Sma I restric-  
tion site had been introduced at the other end of the  
MDV-1 cDNA sequence so that when this plasmid is lin-  
earized by digestion with endonuclease Sma I and then  
incubated with T7 RNA polymerase, transcription termi-  
25 nates two nucleotides before the end of MDV-1 (+) RNA.  
The resulting transcripts, though lacking the natural  
3'-terminal dinucleotide, CpA-OH, serve as excellent  
templates for exponential replication by Q $\beta$  replicase.  
A full description of the construction and use of this  
30 plasmid will be published elsewhere (13).

#### Plasmid Containing a Polylinker within the MDV-1 cDNA Sequence

35 The PpuM I-BstE II fragment of the MDV-1 cDNA sequence  
in pT7-MDV DNA was replaced with the corresponding PpuM  
I-BstE II fragment of a modified MDV-1 cDNA that con-

5       tained a unique Xba I recognition sequence in place of  
the only Hinf I recognition sequence in MDV-1 cDNA  
(21). A synthetic DNA fragment (prepared by annealing  
dCTAGATCTCGAGGCCTG to dCTAGCAGGCCTCGAGAT) was then  
cloned into this Xba I site, converting it into a  
polylinker possessing unique restriction sites for Xba  
I, Bgl II, Xho I, and Stu I. This plasmid, designated  
pT7-MDV-poly, is diagrammed in Figure 1. The RNA syn-  
10       thesized from this plasmid is designated MDV-poly.  
Recombinant RNAs can be generated by inserting any  
heterologous DNA sequence into one of the unique re-  
striction sites in the polylinker of pT7-MDV-poly, and  
then utilizing the resulting plasmid as a template for  
15       transcription by T7 RNA polymerase.

#### Plasmids for Synthesizing Recombinant Probes by Tran- scription

20       pT7-MDV-fal-un was constructed by inserting a synthetic  
probe sequence (prepared by annealing dTCGAGACTAACATA-  
GGTCTTAAGTTGACTAACA to dTCGATGTTAGTCAAGTTAAGACCTATGT-  
TAGTC) into the Xho I site of the polylinker sequence  
in pT7-MDV-poly. pT7-MDV-fal-st was constructed by  
inserting the related synthetic probe sequence (pre-  
25       pared by annealing dTCGAGACTAACATAGGTCTAACTTGTTAGTCA  
to dTCGATGACTAACAAGTTAAGACCTATGTTAGTC) into the site in  
pT7-MDV-poly. Both probe sequences were complementary  
to the unique 21-base-pair sequences motif that is  
repeated over 1,000 times in P. falciparum DNA (14-16).  
30       The specific sequence used herein was determined by  
sequencing cloned repetitive DNA isolated from strain  
FCR-3/Gambia.

#### Nucleotide Sequence Analysis

35       The nucleotide sequence in the recombinant region of  
each plasmid was determined by the chain termination

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procedure (22), utilizing 7-deaza-deoxyguanosine 5'-triphosphate (Boehringer Mannheim) in place of deoxyguanosine 5'-triphosphate (23), and [<sup>35</sup>S]deoxycytidine 5'( $\alpha$ -thio) triphosphate (New England Nuclear) as label (24). Sequencing reactions were carried out on total plasmid DNA (25), utilizing a 20-nucleotide primer (Pharmacia) that was complementary to the T7 promoter sequence (26).

### Transcription

Plasmids were isolated from bacteria by the method of Holmes and Quigley (27) and purified by gel filtration chromatography (28) on Sephacryl S-1000 (Pharmacia). Plasmid DNA was then digested with Sma I or Stu I. Transcription was carried out according to a modification of the protocol of Axelrod and Kramer (29): 1  $\mu$ g of linearized DNA was incubated with 80 units of T7 RNA polymerase for 3 hr at 37°C in 40  $\mu$ l of 400  $\mu$ M ATP, 400  $\mu$ M [ $\alpha$ -<sup>32</sup>P] CTP, 400  $\mu$ M GTP, 400  $\mu$ M UTP, 50 mM Tris-HCl (pH 8.0), 12mM MgCl<sub>2</sub>, 5 mM dithiothreitol (freshly prepared), 100  $\mu$ g/ml bovine serum albumin, 4 mM spermidine, and 1 unit/ $\mu$ l of the ribonuclease inhibitor, RNasin (Promega Biotec). The [<sup>32</sup>P] transcripts were incubated with 0.1  $\mu$ g/ $\mu$ l of ribonuclease-free deoxyribonuclease I (30) to destroy the template DNA, and then purified by phenol/chloroform/isoamyl alcohol extraction, followed by precipitation with ethanol. The concentration of each RNA was determined from its specific radioactivity. The size and homogeneity of the RNA was determined by electrophoresis through 6% polyacrylamide slab gels in the presence of 7 M urea (31). The RNA bands were visualized by silver staining (32).



### Replication

Between  $1.4 \times 10^{-18}$ g and  $1.4 \times 10^{-10}$ g of transcripts (depending on the reaction) were incubated with  $2.4 \times 10^{-6}$ g of Q $\beta$  replicase at 37°C in 25  $\mu$ l of 400  $\mu$ M ATP, 400  $\mu$ M [ $\alpha$ - $^{32}$ P]CTP, 400  $\mu$ M GTP, 400  $\mu$ M UTP, 14 mM MgCl $_2$ , and 90 mM Tris-HCl (pH 7.5). Each reaction was sampled every 5 min. The amount of RNA in each 2- $\mu$ l sample was determined by binding the [ $^{32}$ P]RNA to DE81 cellulose discs (Whatman), as described by Maxwell and his co-workers (33), and measuring the radioactivity on each disc with a scintillation counter. The size and homogeneity of the RNA in selected samples was determined by polyacrylamide gel electrophoresis. The [ $^{32}$ P]RNA bands were detected by autoradiography.

### Hybridization

Two different plasmids were used as targets. The first, pPFR6, was constructed by the insertion of a DNA fragment containing 45 tandem copies of the 21 base-pair repetitive sequence of *P. falciparum* DNA (strain FCR-3/Gambia) into the BamH I site of pUC13 DNA (Boehringer Mannheim). Fifteen of the 45 repeated sequences were identical to the inserted sequence in the recombinant RNA probes. The other repeated sequences contained microheterogeneities. The second plasmid, pUC13, served as a negative control for hybridization of the recombinant RNA probes. Each plasmid was linearized by digestion with BamH I, denatured by incubation in 0.4N NaOH for 60 sec at 42°C, and 0.5- $\mu$ g or 1.0- $\mu$ g aliquots were bound to a BA83 nitrocellulose membrane (Schleicher and Schuell), according to the dot-blot procedure of Kafatos and his co-workers (34). The membrane was cut into sections, each containing a duplicate pair of pPFR6 and pUC13 dot-blots. Each membrane section was prehybridized for 3

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hr at 37°C in 5X SSPE (5X SSPE is 900 mM NaCl, 50 mM sodium phosphate (pH 7.4), and 5 mM EDTA), 2 mg/ml sodium dodecyl sulfate, 500 µg/ml heparin (Sigma), and 20% formamide. Each membrane section was then incubated for 4 hrs at 25°C with between 23 ng and 150 ng of the [<sup>32</sup>P]RNA to be tested and 10 µg of unlabelled E. coli tRNA carrier (Boehringer Mannheim) dissolved in 2 ml of the prehybridization buffer. The following RNAs were tested: MDV-poly, MDV-fal-un, MDV-fal-st, and truncated versions of each (transcribed from plasmids cleaved at the Stu I site). After hybridization, the membranes were washed three times (15 min/wash) at 25°C with a solution containing 4X SSPE, 2 mg/ml sodium dodecyl sulfate, and 400 µg/ml heparin, followed by two 10-min washes at 25°C in 1X SSPE, and a 12-min wash at 37°C in 1X SSPE. The hybridized RNA was detected by autoradiography.

#### 20     Replication of Recombinant RNAs after Hybridization

To study the replicability of bound RNA, MDV-fal-un RNA hybridized to dot-blot was eluted by boiling in 200 µl of water for 60 sec. The integrity of the eluted RNA was analyzed by polyacrylamide gel electrophoresis. The replicability of the eluted RNA was determined by initiating a Qβ replicase reaction with 0.1 µl of the eluant. This reaction was followed kinetically, and the identity of the product RNA was determined by polyacrylamide gel electrophoresis. In addition, individual hybridized dot-blot were added directly to Qβ replicase reactions to see whether bound RNA could serve as a template. These reactions contained 100 µg/ml bovine serum albumin to preclude the possibility that Qβ replicase would bind to the nitrocellulose membrane.

Preparation of Hybridization Reactions

Eight different 70- $\mu$ liter hybridization reactions were prepared containing the following number of molecules of a transcript of the HIV-1 pol region:  $2.5 \times 10^8$ ,  $2.5 \times 10^7$ ,  $2.5 \times 10^6$ ,  $2.5 \times 10^5$ ,  $2.5 \times 10^4$ ,  $2.5 \times 10^3$ ,  $2.5 \times 10^2$ , and 0.  $5 \times 10^8$  molecules of a recombinant RNA (shown in Figure 2A) containing a probe sequence that is complementary to a conserved section of the pol region of HIV-1 mRNA was added to each of the eight reactions and hybridized to the target molecules in the presence of 2.5 M guanidine thiocyanate. The resulting probe-target hybrids were isolated by three cycles of reversible target capture on magnetic beads, as described on page 36 of the proposal. The recombinant-RNA probes were then released from the isolated hybrids by incubation in 50  $\mu$ liters of a salt-free buffer at 37°C. 40  $\mu$ liters of each of these eight solutions was used to initiate eight corresponding 120- $\mu$ liter Q $\beta$  replicase amplification reactions. These reactions were incubated in parallel. 5- $\mu$ liters samples of each of the eight reactions were withdrawn at two-minute intervals between 6 and 28 minutes. The concentration of magnesium in these reactions (7 mM) was half of that usually used, because we recently discovered that at lower magnesium concentrations the rate of replication of contaminating MDV-1 RNA is suppressed compared with the rate of replication of recombinant RNA. A portion of the RNA contained in each of the 96 samples was analyzed by acrylamide gel electrophoresis and shown to contain only recombinant RNA, confirming the utility of using a lower magnesium concentration. Finally, a portion of the RNA contained in each of the 96 samples (corresponding to a 1- $\mu$ liter aliquot of the amplification reaction) was precipitated in 3.5% phosphoric acid and bound to a nylon membrane in a 96-

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position dot-blot format.

### Experimental Results

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#### Design of the Replicatable Probes

One of the goals of the present invention was to construct an RNA that would serve both as a specific probe for P. falciparum DNA and as a template for exponential amplification by Q $\beta$  replicase. MDV-1 RNA was chosen as the parent molecule because modified MDV-1 RNAs can be synthesized by transcription from recombinant plasmids (13). MDV-1 RNA contains many stable secondary structures (35-37), and these secondary structures are required for replication (38-40). The site which was chosen for inserting probe sequences into MDV-1 RNA was located on the exterior of the molecule (12), where the insert was less likely to disturb the structure, and therefore less likely to interfere with replication. In selecting the sequence of the probe two main concerns had to be reconciled: 1) the inserted sequence might have to assume a single-stranded conformation for it to hybridize to its target sequence; and 2) the inserted sequence might have to form secondary structures, otherwise the product and the template might form a lethal duplex during replication (40). Because these two concerns imposed contradictory constraints on the design of the inserted sequence, two recombinant RNAs were prepared each containing a different probe sequence. The first recombinant, MDV-fal-un RNA, was likely to possess an unstructured probe region, according to a computer analysis of its sequence (41). The second recombinant, MDV-fal-st RNA, differed from MDV-fal-un RNA in a 5-nucleotide region. As a consequence, it was likely to form a more stable secondary structure in the region

containing the probe. Figure 2 shows the nucleotide sequence and probable secondary structure (41) of MDV-fal-un RNA, MDV-fal-st RNA, and MDV-poly RNA.

5

#### Replication of the Recombinant RNAs

Four different RNAs were prepared by transcription in vitro: MDV-1, MDV-poly, MDV-fal-un, and MDV-fal-st. Electrophoretic analysis of these transcripts, as illustrated in Figure 3(A), demonstrated that the recombinants that contained probe sequences could be distinguished easily from the other transcripts by their relative mobility. The transcripts were then isolated from the reaction mixture and used as templates for the synthesis of additional RNA by Q $\beta$  replicase. Kinetic analysis of the amount of RNA synthesized in the Q $\beta$  replicase reactions, as shown in Figure 3(B), demonstrated that both the structured and the unstructured recombinant RNAs were excellent templates for exponential replication. Furthermore, as shown in Figure 3(C), electrophoretic analysis of the RNA synthesized in each Q $\beta$  replicase reaction, showed that the products were homogeneous replicates of the original transcripts.

25

To compare the rate of replication of recombinant RNA to the rate of replication of MDV-1 RNA, Q $\beta$  replicase reactions were initiated with mixtures of MDV-fal-st (+) RNA and MDV-1 (+) RNA. The reactions were incubated for 20 minutes, permitting the synthesis of approximately ten million copies of each RNA. The RNA products of the reactions were analyzed by polyacrylamide gel electrophoresis. The results are shown in Table 1 and indicate that, despite recombinant RNA being 55 nucleotides longer than the MDV-1 RNA from which it was derived, it is amplified at essentially the same rate

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by Q $\beta$  replicase.

5 Table 1. Replication of Mixtures of MDV-1 RNA and Recombinant RNA

100	Initial RNA (fg)		Product RNA (ng)	
	MDV-1	MDV-fal-st	MDV-1	MDV-fal-st
	1	1	42	54
15	10	1	115	13
	1	10	17	149

20 Exponential Amplification after Serial Dilution

Because the sensitivity of bioassays employing replicatable probes will depend on how few recombinant molecules can serve as templates for the synthesis of an easily detectable quantity of product RNA, a series of Q $\beta$  replicase reactions were initiated with decreasing amounts of MDV-fal-un RNA. The results of this series of reactions are shown in Figure 4 and demonstrate that a reaction initiated with as few as 1,000 molecules of recombinant RNA (0.14 fg) produced 129 nanograms of recombinant RNA product after 30 minutes of synthesis, which is a one-billion-fold amplification. Virtually identical kinetic results were obtained in a parallel series of reactions initiated with MDV-fal-st RNA, indicating that the relative lack of secondary structure in the probe region of MDV-fal-un RNA had no discernible effect on replication.

Electrophoretic analysis showed that the product of the reaction to which no exogenous template had been added was MDV-1 RNA. This analysis is illustrated in the inset of Figure 4. This was expected, because the method which was used for isolating Q $\beta$  replicase (17) does not eliminate all of the contaminating MDV-1 RNA molecules (5). Based on the time it took for this reaction to achieve saturation (when the number of RNA molecules equals the number of active replicase molecules), it is calculated that only 40 molecules of MDV-1 RNA were present initially. This was confirmed by the following results: when a reaction was initiated with 100 molecules of MDV-fal-un RNA, the 25-minute product contained 30% MDV-1 RNA; and a reaction initiated with only 10 molecules of MDV-fal-un RNA contained mostly MDV-1 RNA. Had Q $\beta$  replicase that had been freed of all contaminating RNA (43) been used instead, it would have been expected that only recombinant RNA would have been synthesized.

#### Hybridization of the Recombinant RNAs

Each of the recombinant RNAs was shown to bind specifically to denatured plasmids containing a known quantity of P. falciparum target sequences (Figure 5). Neither recombinant RNA bound to control plasmids that lacked P. falciparum sequences. Furthermore, MDV-poly RNA, which does not contain a probe sequence, did not bind to either plasmid. Taken together, these results demonstrate that both recombinant RNAs bind specifically to their targets and that, under the hybridization conditions employed, the presence of structure in the probe region has little effect on hybridization.

In another experiment, truncated versions of each recombinant probe were prepared by transcription from plasmids that had been linearized by digestion with Stu I, instead of Sma I. These transcripts were 111-nucleotides long, and contained 63 nucleotides from the 5' end of MDV-1 (+) RNA and 48 nucleotides from the insert region, including the entire probe sequence. Under the hybridization conditions employed, no difference could be observed between the hybridization of the truncated RNAs and the hybridization of the full-length RNAs. This result demonstrates that the constrained topology of the MDV-1 domain (44) that surrounds the probe region in the full-length molecule has little effect on the ability of the probe to hybridize to its target.

#### Replication of Recombinant RNAs after Hybridization

Additional experiments were carried out to determine whether hybridized recombinant RNAs retain their ability to serve as templates for Q $\beta$  replicase after all the manipulations that occur during filter hybridization. MDV-fal-un RNA that had been hybridized to plasmids containing P. falciparum sequences was eluted from the dot-blot by a brief heating step. Electrophoretic analysis of this RNA showed that intact recombinant molecules had been released. A portion of the eluted RNA was then tested to see whether it could serve as a template for Q $\beta$  replicase. A ten-million-fold amplification occurred in 20 minutes. Electrophoretic analysis of the product of this reaction showed that it was pure MDV-fal-un RNA. From the results of these experiments it was estimated that at least 10 percent of the eluted RNA had retained its biological activity.



containing hybridized MDV-fal-un RNA were incubated directly with Q $\beta$  replicase. Reactions containing membranes with RNA hybridized to target sequences synthesized more recombinant RNA than reactions containing membranes that lacked target sequences. These preliminary results suggest that a special release step may not be necessary to initiate the replication of hybridized recombinant RNAs.

#### Results of Hybridization Reactions

The results are shown in Figure 7.

#### Experimental Discussion

In most bioassays that employ molecular probes, the amount of probe bound is proportional to the number of targets. Amplification schemes, such as those employing enzymatic reporter groups (45) or polymerase chain reactions (46-48), enable a small number of targets to be detected. However, the relationship between the size of the amplified signal and the number of targets is still linear. The unique kinetic characteristics of Q $\beta$  replicase reactions should permit the design of bioassays in which the size of the amplified signal is linearly proportional to the logarithm of the number of targets. Figure 6 utilizes data from the experiment shown in Figure 4 to illustrate this relationship: the logarithm of the number of recombinant RNA molecules initially present in each reaction was plotted against the amount of RNA synthesized in 25 minutes (by which time each reaction had completed the exponential phase of synthesis). The amount of recombinant RNA initially added to those reactions can be thought of as representing the amount of probe that would have been bound to targets had this been an actual assay, and the

amount of amplified RNA at 25 minutes simulates the signal that would have been detected. The results show that the size of the amplified signal would be in the  
5 hundred-nanogram range, whether the number of targets was as small as one thousand or as large as one billion. Thus, bioassays employing amplification by Q $\beta$  replicase should be able to detect targets over a range of at least six orders of magnitude.

10 Theoretically, assays employing Q $\beta$  replicase should be extremely sensitive, because such reactions can be initiated with as little as one molecule of template RNA (10). In practice, the sensitivity of assays will  
15 be limited by the level of persistence of nonspecifically bound probes. In the experiments described above it was found that 600,000 molecules of MDV-fal-un RNA had bound nonspecifically to nitrocellulose dot-blots that contained 1.5  $\mu$ g of pUC13 DNA. It is appar-  
20 ent that a different hybridization technique will be required to test the limits of sensitivity of assays employing replicatable probes. There are a number of promising methods that should result in a markedly  
25 reduced background, including those where hybridization occurs in solution (49-51) and those that employ "sandwich" hybridization techniques (52,53). By combining an effective background reduction technique with the enormous amplification inherent in the use of replicatable probes, it should be possible to develop assays  
30 that can detect a few hundred molecules of target. This would permit the detection of relatively rare RNA or DNA molecules in a research sample and the detection of even a single infectious agent in a clinical sample.

Replicatable recombinant RNAs containing a variety of inserted sequences have recently been constructed. This leads one to believe that, by choosing appropriate inserts, recombinant RNA probes can be prepared that will be able to detect the nucleic acid of any virus, bacterium, or eukaryotic parasite.

Amplified recombinant RNAs contain a sequence (the probe) that identifies which target was detected. It should therefore be possible to design diagnostic assays that utilize a mixture of recombinant RNAs, each containing a probe sequence specific for the genome of a different infectious agent. After amplification with Q $\beta$  replicase, the RNA population would contain replicates of only those probes that had bound to their targets. Subsequent hybridization of these amplified RNAs to a membrane containing an ordered array of DNA dot-blot complementary to each of the probes would permit the simultaneous identification of several different organisms in the same sample.

The density of each spot in Figure 7 indicates the amount of RNA that is present at each time point. Although no RNA can be seen during the early time points of each reaction, this is the period in which the RNA population is increasing exponentially. The RNA becomes visible at about the same time that the number of RNA molecules equals the number of active replicase molecules (the "saturation point"). Consequently, the amount of RNA in the visible time points increases linearly. The results show that the time at which the saturation point occurs in each amplification reaction is a function of the number of target molecules that were originally present in the corresponding hybridization reaction. The less target

5 molecules that were present, the less the number of  
probes that were bound to targets, and thus, the less  
the number of probes that were available to initiate  
the amplification reaction. This result demonstrates  
that an automated assay could be developed, in which a  
detection device (based, perhaps, on the fluorescence  
of an RNA ligand, such as ethidium bromide) could be  
used to determine the time of saturation. The number  
10 of probes originally present in the amplification  
reaction (which would be proportional to the number of  
targets in the sample) could then be calculated from  
the time it took to reach saturation. The results also  
15 demonstrate an alternative way to determine the number  
of targets present in the sample. The amount of RNA  
present at a particular time point, after all reactions  
have achieved saturation (for example, at 24 minutes),  
is linearly proportional to the logarithm of the number  
of probes originally present in each amplification  
20 reaction (see also Figure 6).

Finally, the results of this improved assay show that  
the current limit of sensitivity is between 2,500 and  
25,000 molecules of target. Reactions initiated with  
25 probes released from hybridization reactions that  
contained a small number of targets gave virtually  
indistinguishable results. Since the RNA that grew out  
of these reactions was recombinant RNA, the released  
RNA must have mainly consisted of non-specifically  
30 hybridized probes that were not removed during the  
hybrid isolation procedure.

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What is claimed is:

- 5 1. A replicatable and hybridizable recombinant single-stranded RNA probe molecule comprising:
  - (a) a recognition sequence for the binding of an RNA-directed RNA polymerase;
  - 10 (b) a sequence required for the initiation of product strand synthesis by the polymerase; and
  - 15 (c) a heterologous RNA sequence inserted at a specific site in the internal region of the recombinant molecule and complementary to an oligo- or polynucleotide of interest.
- 20 2. A recombinant-RNA probe molecule of claim 1, wherein the recognition sequence for the binding of an RNA-directed RNA polymerase is in an internal region of the molecule.
- 25 3. A recombinant-RNA probe molecule of claim 1, wherein the insertion site is not near any sequence required for the binding of the RNA polymerase or for the initiation of product strand synthesis.
- 30 4. A recombinant-RNA probe molecule of claim 1, wherein the insert has a minimal affect on the replicability of the molecule.
- 35 5. A recombinant-RNA probe molecule of claim 3, wherein the insert has a minimal effect upon the secondary and tertiary structure of the mole-

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cule.

- 5 6. A recombinant RNA probe molecule of claim 1, wherein the sequence required for the initiation of product strand synthesis is a cytidine-rich 3'-terminal sequence.
- 10 7. A recombinant-RNA probe molecule of claim 1, wherein the RNA-directed RNA polymerase is Q $\beta$  replicase.
- 15 8. A recombinant-RNA probe molecule of claim 1, wherein the specific insertion site is at a specific nucleotide.
- 20 9. A recombinant-RNA probe molecule of claim 1, wherein the molecule is a variant RNA template for Q $\beta$  replicase or a mutant thereof.
- 25 10. A recombinant-RNA probe molecule of claim 9, wherein the variant RNA template is MDV-1 RNA or a mutant thereof.
- 30 11. A recombinant-RNA probe molecule of claim 10, wherein the the MDV-1 RNA is MDV-1 (+) RNA.
12. A recombinant-RNA probe molecule of claim 10, wherein the MDV-1 RNA is MDV-1 (-) RNA.
- 35 13. A recombinant-RNA probe molecule of claim 11, wherein the heterologous sequence of interest is inserted between nucleotides 63 and 64.
14. A recombinant-RNA probe molecule of claim 1, wherein the molecule is a transcript derived

from a recombinant plasmid by incubation with a DNA-directed RNA polymerase.

- 5      15.      A recombinant-RNA probe molecule of claim 14,  
         wherein the molecule is a variant RNA template  
         for Q $\beta$  replicase or a mutant thereof.
- 10      16.      A recombinant-RNA probe molecule of claim 15,  
         wherein the molecule is MDV-1 RNA or a mutant  
         thereof.
- 15      17.      A recombinant-RNA probe molecule of claim 16,  
         wherein the MDV-1 RNA is MDV-1 (+) RNA.
18.      A recombinant-RNA probe molecule of claim 16,  
         wherein the MDV-1 RNA is MDV-1 (-) RNA.
- 20      19.      A recombinant-RNA probe molecule of claim 17,  
         wherein the heterologous sequence is inserted  
         between nucleotides 63 and 64.
- 25      20.      A recombinant-RNA probe molecule of claim 1,  
         wherein the inserted heterologous sequence is  
         complementary to a specific nucleic acid se-  
         quence of an infectious agent.
- 30      21.      A recombinant-RNA probe molecule of claim 20,  
         wherein the infectious agent is a virus.
22.      A recombinant-RNA probe molecule of claim 20,  
         wherein the infectious agent is a viroid or  
         virusoid.
- 35      23.      A recombinant-RNA probe molecule of claim 20,  
         wherein the infectious agent is a prokaryote.

24. A recombinant-RNA probe molecule of claim 23,  
wherein the prokaryote is a bacterium.
- 5 25. A recombinant-RNA probe molecule of claim 20,  
wherein the infectious agent is a eukaryote.
26. A recombinant-RNA probe molecule of claim 25,  
wherein the eukaryote is a parasitic protozoan.
- 10 27. A recombinant-RNA probe molecule of claim 26,  
wherein the parasitic protozoan causes malaria.
- 15 28. A recombinant-RNA probe molecule of claim 1,  
wherein the inserted heterologous sequence is  
complementary to a specific gene sequence or  
portion thereof.
- 20 29. A recombinant-RNA probe molecule of claim 28,  
wherein the specific gene sequence is an allele  
or portion thereof.
- 25 30. A method for determining the presence or concen-  
tration of an oligo- or polynucleotide of inter-  
est in a sample, comprising the steps of:
- 30 (a) forming a specific complex between the  
recombinant-RNA probe molecule of claim 1  
and the oligo- or polynucleotide of  
interest, by incubating the sample with  
the recombinant-RNA probe molecules un-  
der suitable conditions and for a suf-  
35 ficient period of time to permit comple-  
mentary nucleotide sequences to hybrid-  
ize;

- 5 (b) removing unhybridized recombinant-RNA probe molecules from the reaction mixture;
- 10 (c) incubating the reaction mixture with an RNA-directed RNA polymerase capable of synthesizing additional copies of the recombinant-RNA probe molecules that are hybridized to the oligo- or polynucleotide of interest; and
- 15 (d) detecting the recombinant-RNA probe molecules synthesized in step (c), thereby determining the presence or concentration of the oligo- or polynucleotide of interest.
- 20 31. A method of claim 30, wherein the oligo- or polynucleotide in the sample is bound to a solid support.
- 25 32. A method of claim 31, wherein the solid support is a nitrocellulose or nylon membrane.
- 30 33. A method of claim 30, wherein in step (a) the oligo- or polynucleotide of interest and the recombinant-RNA probe molecule are in solution.
- 35 34. A method of claim 33, wherein in step (b) the hybridized recombinant-RNA probe molecules are separated from the unhybridized probe molecules through the capture of the oligo- or polynucleotide onto a solid support.



35. A method of claim 30 wherein detecting is carried out by the incorporation of radioactively labelled ribonucleoside 5'-triphosphate precursors into the recombinant-RNA products.
36. A method of claim 30 wherein detecting is carried out by the incorporation of chemically modified ribonucleoside 5'-triphosphate precursors into the recombinant-RNA products.
37. A method of claim 36, wherein the chemically modified ribonucleoside 5'-triphosphate precursors are biotinylated.
38. A method of claim 36, wherein the chemically modified ribonucleoside 5'-triphosphate precursors are fluorescent.
39. A method of claim 30, wherein detecting is carried out by the binding of RNA-specific chromogenic or fluorogenic dyes to the recombinant-RNA products.
40. A method of claim 30, wherein detecting is carried out by physical methods.
41. A method of claim 30, wherein in step (c) the RNA-directed RNA polymerase is Q $\beta$  replicase.
42. Recombinant-RNA probe molecules produced by the method of claim 30.
43. Recombinant-RNA probe molecules produced by the method of claim 41.

44. A method of claim 30, wherein the time of incubation in step (c) is sufficiently short so that the number of recombinant-RNA product strands does not exceed the number of polymerase molecules, with the result that the number of recombinant-RNA product molecules is linearly proportional to the number of recombinant-RNA probe molecules originally hybridized.
45. A method of claim 30, wherein the time of incubation in step (c) is sufficiently long so that the number of recombinant-RNA product strands exceeds the number of polymerase molecules, with the result that the number of recombinant-RNA product molecules is proportional to the logarithm of the number of recombinant-RNA probe molecules originally hybridized.
46. A method for simultaneously determining the presence or concentration of several different oligo- or polynucleotides of interest in a sample, comprising the steps of:
- (a) forming specific complexes between a mixture of different types of recombinant-RNA probe molecules of claim 1, each type having a different inserted sequence, and the oligo- or polynucleotides of interest, by incubating the sample with the mixture of recombinant-RNA probe molecules under suitable conditions and for a sufficient period of time to permit complementary nucleotide sequences to hybridize;

- 5
- (b) removing unhybridized recombinant-RNA probe molecules from the reaction mixture;
- 10
- (c) incubating the reaction mixture with an RNA-directed RNA polymerase capable of synthesizing additional copies of the recombinant-RNA probe molecules that are hybridized to the oligo- or polynucleotides of interest;
- 15
- (d) separating the mixture of synthesized recombinant-RNAs by hybridizing them to an ordered array of polynucleotides bound to a membrane, where each of the polynucleotides is complementary to one type of synthesized recombinant-RNA; and
- 20
- (e) detecting the recombinant-RNA probe molecules produced in step (d), thereby determining the presence or concentration of each oligo- or polynucleotide of interest.

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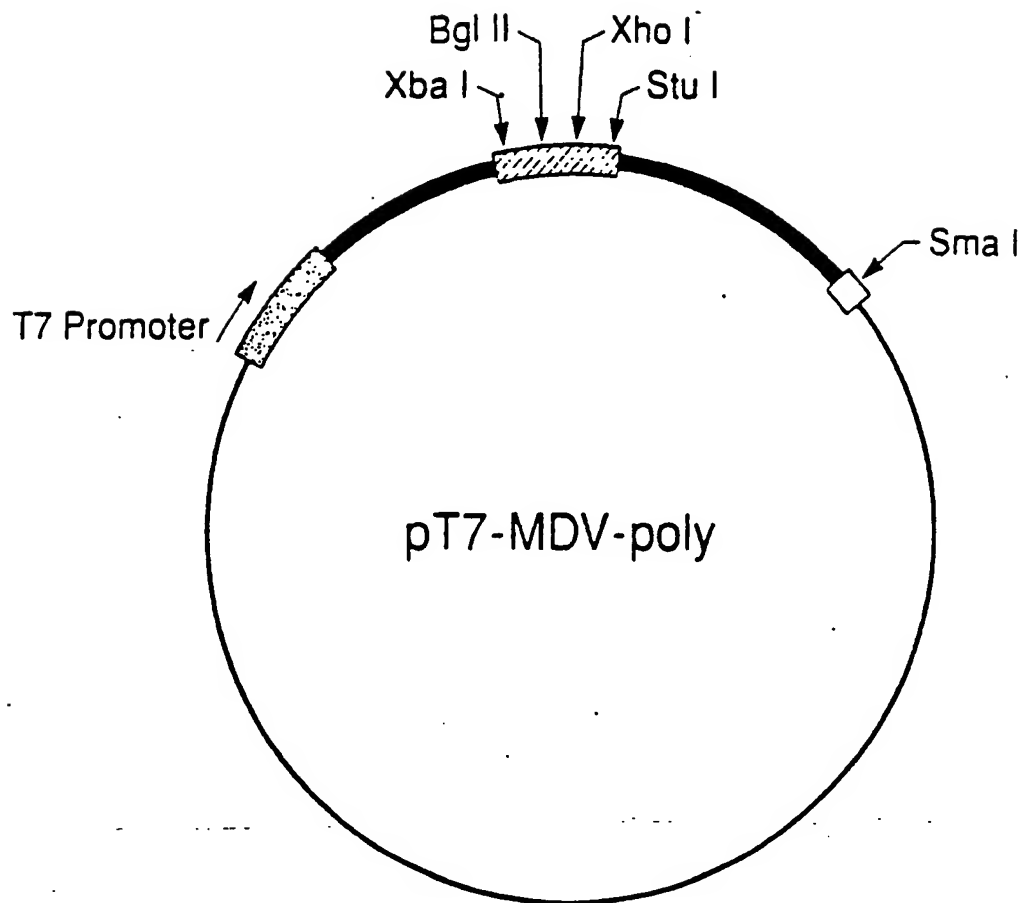


Figure 1

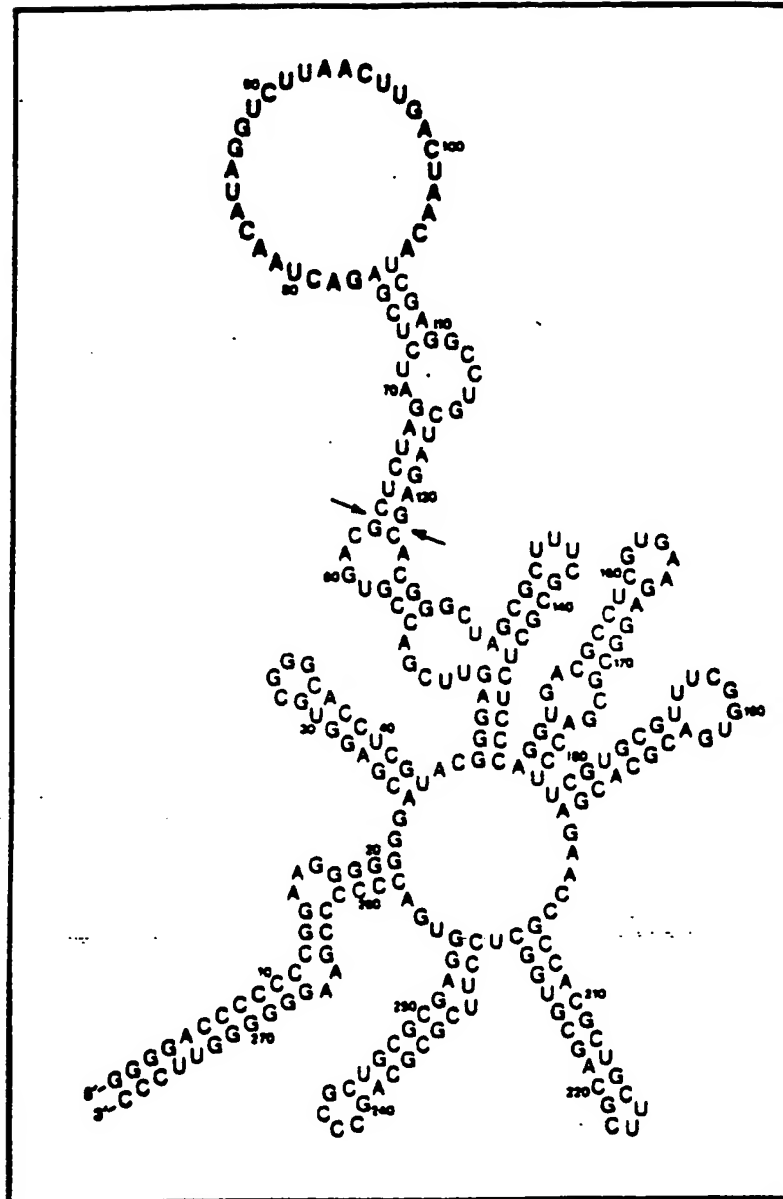
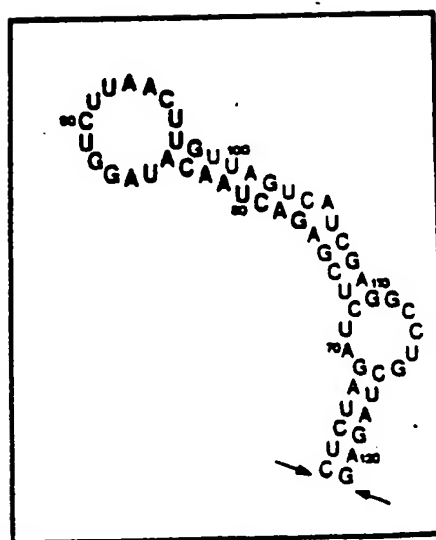


Figure 2(A)

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**Figure 2(B)**

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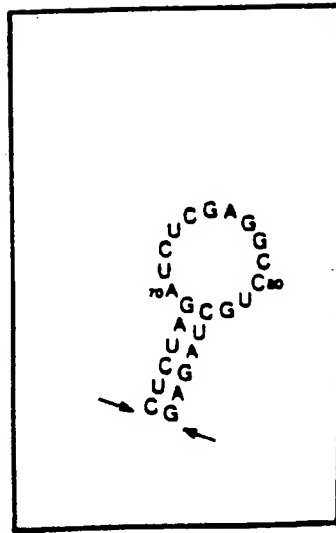


Figure 2(C).

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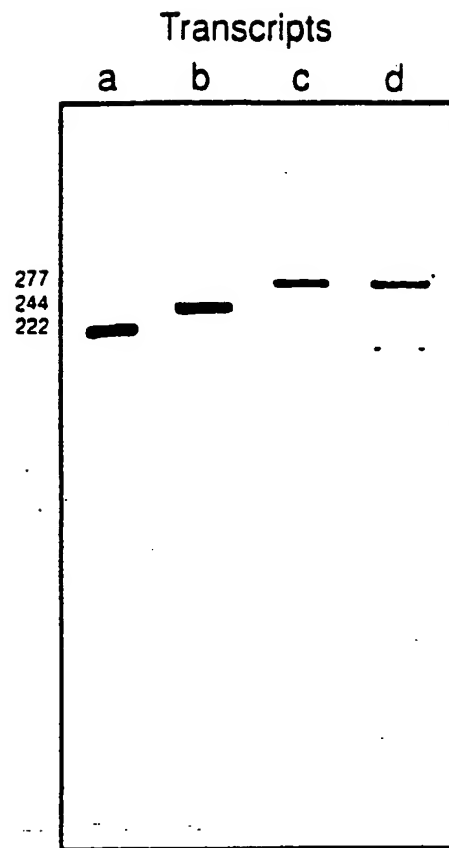


Figure 3(A)



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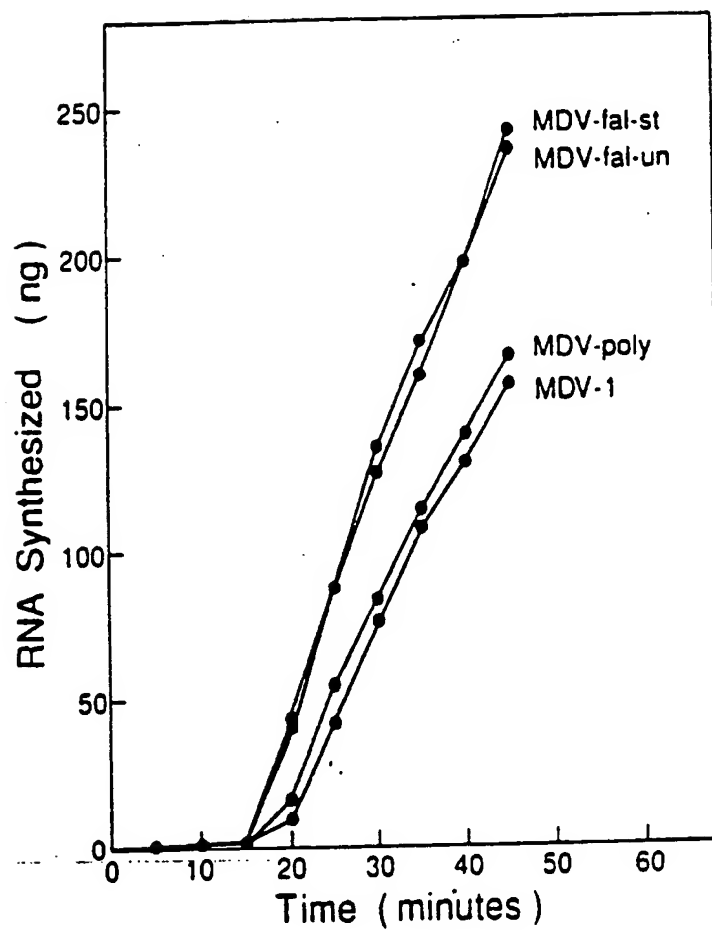


Figure 3(B)

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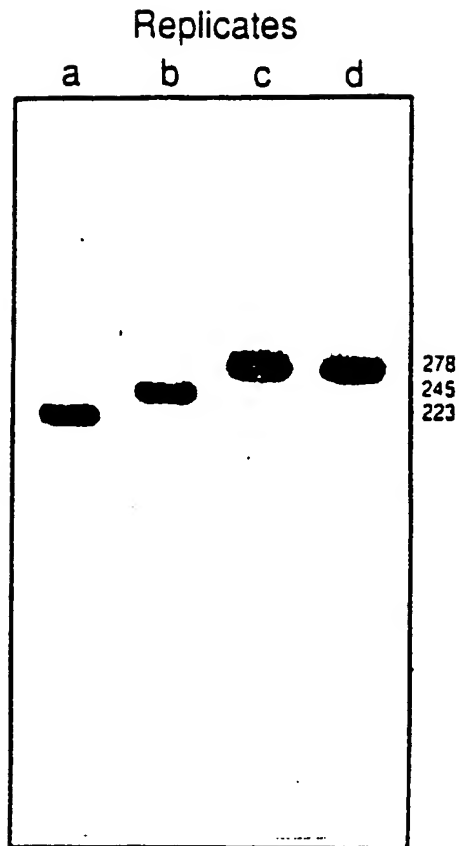


Figure 3(C)

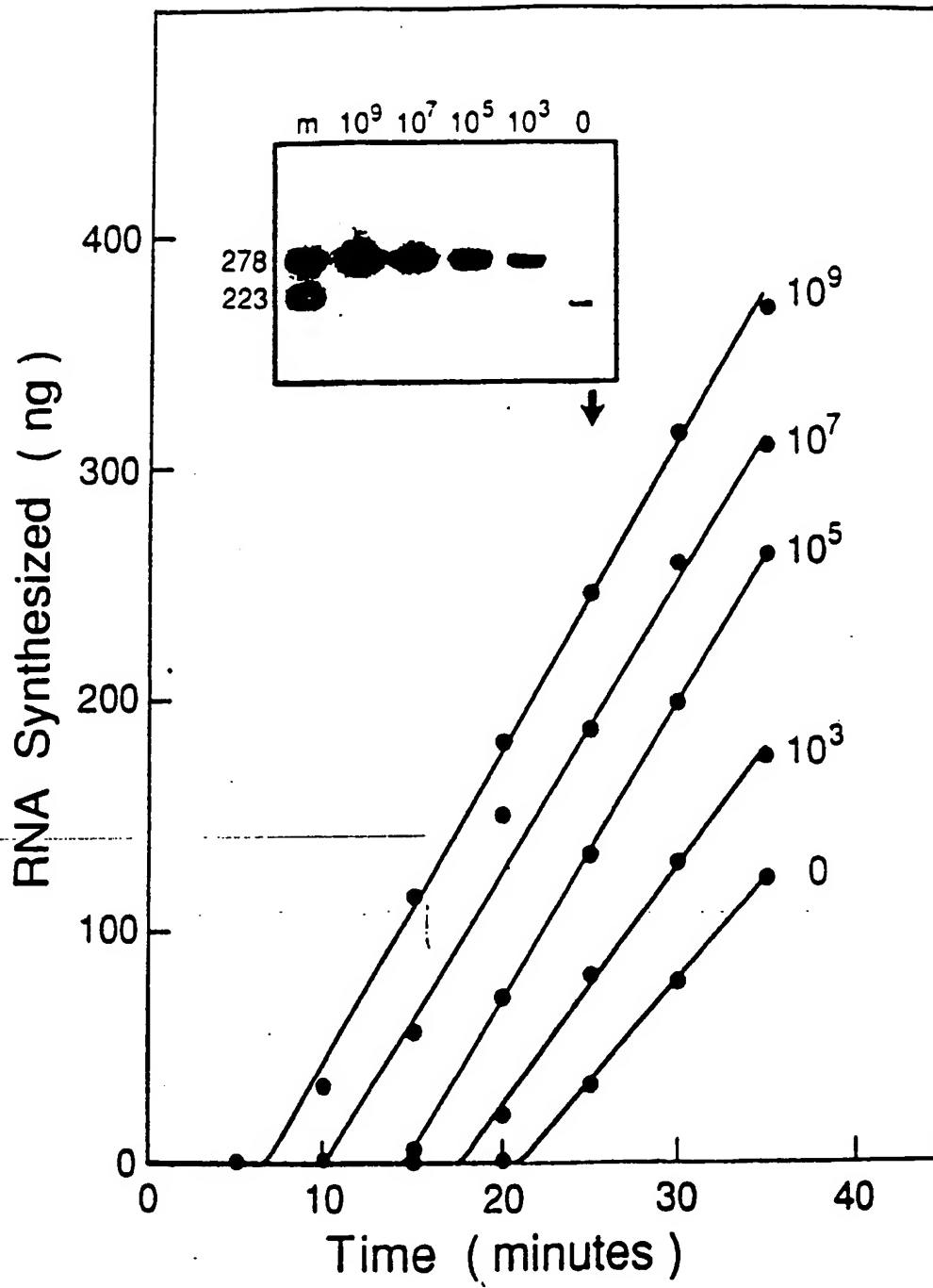


Figure 4

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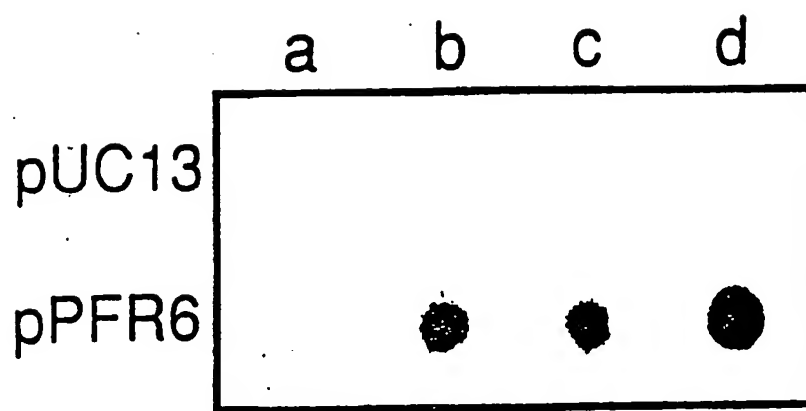


Figure 5

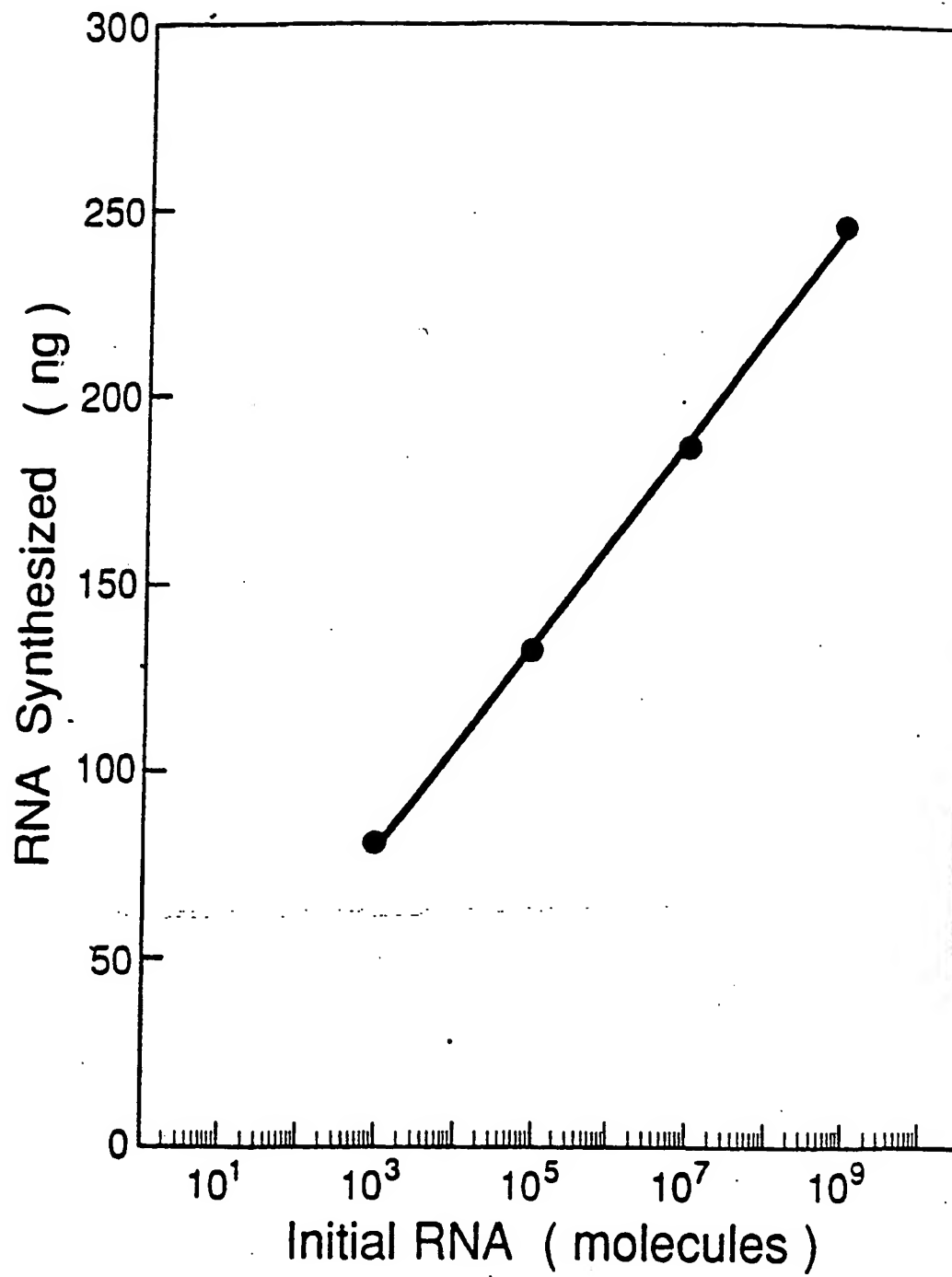


Figure 6

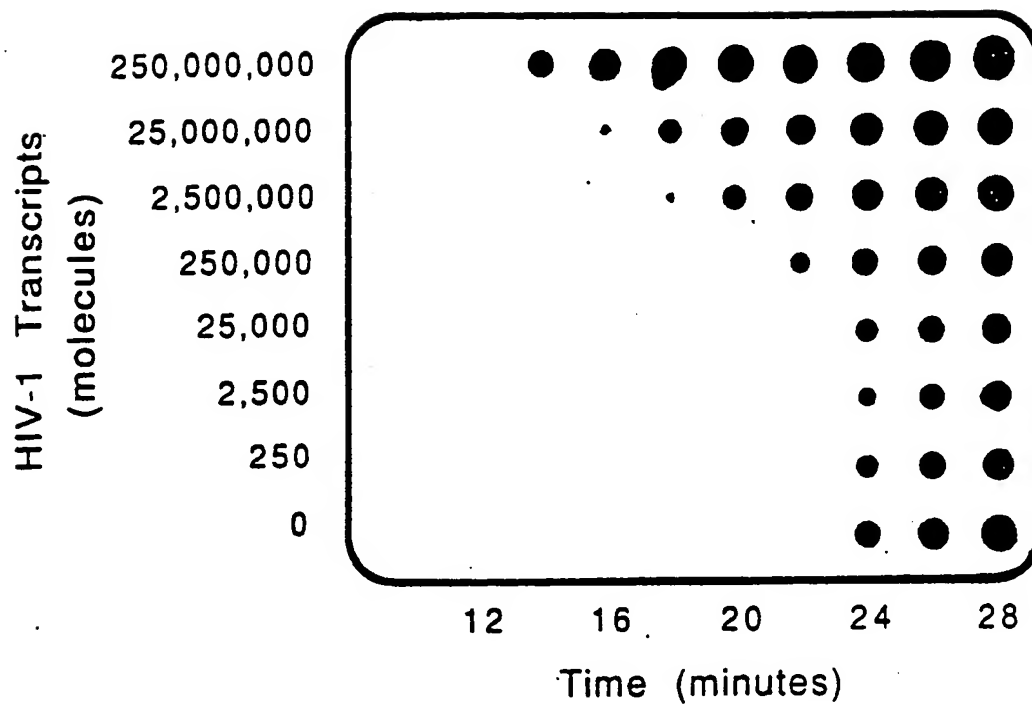


Figure 7

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/01673**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
INT. CL. (4): C12Q 1/68; C07H 21/00		
U.S. CL.: 435/6; 536/27		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	435/6, 91, 5, 29, 803 436/501, 800 935/78; 536/27	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
COMPUTER SEARCHES: APS, CA SEARCH, WORLD PATENTS INDEX (WPI, WPIL), MEDLINE		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
P, X/Y	U.S., A, 4,786,600 (KRAMER ET AL) 22 Nov. 1988, see abstract, column 5, lines 1-31; column 7, line 59 through column 8, line 25 column 9, lines 1-29 and 40-60; column 16, lines 16-62 and column 18, lines 1-68.	1-29, 42 43/30-41 44-46
X/Y	Journal of Molecular Biology, Volume 171, issued 15 Dec. 1983, E. A. Miele et al, "Autocatalytic Replication of a Recombinant RNA, see abstract, pages 281, 282, 285 and 286.	1-19, 28 29, 42, 43/20-27 30-41, 44-46
Y	U.S., A, 4,563,417 (ALBARELLA ET AL) 07 Jan. 1986, see column 11, line 67 through column 12, line 52.	33, 34
P,Y	U.S., A, 4,749,647 (THOMAS ET AL) 07 June 1988, see column 19, lines 29-41 and column 20, lines 8-29.	1-46
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <b>02 JUNE 1989</b>		Date of Mailing of this International Search Report <b>05 JUL 1989</b>
International Searching Authority <b>ISA/US</b>		Signature of Authorized Officer <b>JEREMY M. JAY</b> <i>Jeremy M. Jay</i>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
.Y	U.S., A, 4,683,195 (MULLIS ET AL) 28 Jul. 1987, see abstract; column 18, lines 13-38; column 19, lines 41-48.	1-46
P,Y	Chemical Abstracts, Volume 109, No. 19, issued 07 Nov. 1988, Chu, B. et al, "Repliative RNA reporter systems for signal amplification in biopolymer assays", see page 387, column 1, the abstract no. 166862b, PCT Int. Appl. WO 87 06,270.	1-46
P,Y	Chemical Abstracts, Volume 109, No. 25, issued 19 Dec. 1988, Lizardi, P. M. et al, "Exponential amplification of recombinant-RNA hybridization probes", Bio/Technology 1988 6(10), 1197-202 (Eng).	1-46